PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/68, C12P 19/34, C12N 1/12, C07K 13/00, C07H 21/02, 21/04, 19/04

A1

(11) International Publication Number: **WO 99/40223**

(43) International Publication Date: 12 August 1999 (12.08.99)

(21) International Application Number: PCT/US99/02104

(22) International Filing Date: 2 February 1999 (02.02.99)

(30) Priority Data:
09/018,695 4 February 1998 (04.02.98) US

(71) Applicant: AMERSHAM PHARMACIA BIOTECH, INC. [US/US]; 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327 (US).

(72) Inventors: KUMAR, Shiv; 5093 Stansbury Drive, Solon, OH 44139 (US). NAMPALLI, Satyam; 1414 SOM Center Road, Mayfield Heights, OH 44124 (US). McARDLE, Bernard, F.; 1732 Stone Creek Lane, Twinsburg, OH 44087 (US). FULLER, Carl, W.; 3397 E. Monmouth Road, Cleveland, OH 44118 (US).

(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: DIDEOXY DYE TERMINATORS

(57) Abstract

A kit for DNA sequencing comprising a first, second, third and fourth dye terminator molecules, each of the dye terminator molecules comprising a dye molecule, a linker of at least 10 atoms in length and either ddATP, ddCTP, ddGTP or ddTTP as a mono or tri-phosphate and a thermostable DNA polymerase.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

DESCRIPTION

DIDEOXY DYE TERMINATORS

FIELD OF THE INVENTION

This invention relates to dye terminator nucleic acid sequencing and reagents for such sequencing.

BACKGROUND OF THE INVENTION

The following is a discussion of the relevant art, none of which is admitted to be prior art to the appended claims.

Sequence reaction products must be labeled. This can be done using labeled primers, labeled nucleotides (usually radioactive dNTPs) or labeled ddNTP terminators. The use of labeled terminators has the advantage of leaving false-stops undetectable.

DNA sequence bands do not necessarily have uniform intensities. It is useful to express band intensity variability numerically. This can be done by reporting the ratio of maximum to minimum intensity of nearby bands (within a window of perhaps 40 bases)in a DNA sequence or, with normalization and correction for systematic "drift" in intensity by reporting the root mean square of band intensities (typically peak heights)(Fuller, C.W., *Comments* 16(3):1-8, 1989). It is advantageous to have uniformity of band intensity as sequence accuracy and read-length is improved with bands of more uniform intensity.

For accurate reading, the mobility of any given sequencing reaction product must migrate through the electrophoresis gel with a speed proportional only to its length. Products which migrate faster or slower than normal for a given length will result in sequence ambiguities or errors known as "compressions".

Anomalous migration speed can be caused by secondary structure of the DNA and is apparently the cause of most "compression" artifacts seen in radioactive-label (and other) sequencing experiments at GC-rich regions. These can often be resolved by the use of analogs of dGTP such as 7-deaza-dGTP or dITP. Another compression-like artifact is observed when some dye-labeled ddNTPs are used for sequencing. Several examples of this can be seen in Lee, L.G., Connell, C.R., Woo, S.L., Cheng, R.D., McArdle, B.F., Fuller, C.W., Halloran, N.C., and Wilson. R.K., *Nucleic Acids Res.*, 20:2471-2483, 1992 (see figures 4g, 4h and 6h using ddCTP labeled with tetramethylbodipy and TMR or ddGTP labeled with bifluor). These compression-like artifacts are produced, even in sequences which are compression-free when sequenced radioactively or with dye-labeled primers. These artifacts can sometimes be eliminated by substituting dITP for dGTP or alpha-thio dNTPs for normal dNTPs (Lee, L.G. et al., *Nucleic Acids Res.*, 20: 2471-2483, 1992; U.S. Patent No. 5,187,085). Similar artifacts seen with the fluorescein dye-labeled ddNTPs sold by Applied Biosystems for dye-terminator sequencing with T7 DNA polymerase are resolved by substituting alpha-thio dNTPs for normal dNTPs (Lee,

L.G. et al., *Nucleic Acids Res.*, 20: 2471-2483, 1992; U.S. Patent No. 5,187,085).

Prober, J.M., Trainor, G.L., Dam, R.J., Hobbs, F.W., Robertson, C.W., Zagursky, R.J., Cocuzza, A.J., Jensen, M.A. and Baumeister, K., *Science* 238:336-41 (1987) performed sequencing using terminators labeled with substituted succinyl-fluoresceins with linkers of 10 atoms in length, together with dATP, dCTP, dTTP, 7-deaza-dGTP and AMV reverse transcriptase, and a fluorescence-detecting instrument. From Fig. 6 of this paper is clear that overall band intensities varied by more than 10-fold, far more than the best available current methods with dye primers or radioactive labels.

Dideoxy NTP terminators that have the same basic structure as the Prober *et al.* (1987) terminators, but have four rhodamine dyes used in place of the succinyl fluoresceins and linkers of 5 atoms in length, have been used for sequencing with Taq polymerase. In order to use these terminators, dITP is used in place of dGTP or 7-deaza-dGTP to eliminate severe "compression" artifacts. This method has been practiced using cloned Taq DNA polymerase(Bergot, WO 9105060; Parker, L.T., Deng, Q, Zakeri, H., Carlson, C. Nickerson, D.A., Kwok, P.Y., *Biotechniques* 19(1):116-121, 1995) and with a mutant of Taq polymerase (D49G, AmpliTaq CS) lacking 5'-3' exonuclease activity. However, band intensities vary by as much as 20-fold, limiting the accuracy and read-length possible with the method (Parker, L.T., Zakeri, H., Deng, Q., Spurgeon, S., Kwok, P.Y., Nickerson, D.A., *Biotechniqu s* 21(4):694-699, 1996).

Lee, L.G., Connell, C.R., Woo, S.L., Cheng, R.D., McArdle, B.F., Fuller, C.W., Hallorand, N.D. and Wilson, R.K., *Nucleic Acids Res.*, 20:2471,1992)describe sequencing with a set of ddNTP terminators and T7 DNA polymerase. All have fluorescein-type dyes attached to the ddNTPs in essentially the same manner as the rhodamine terminators used for Taq sequencing. These are used with modified T7 DNA polymerase (Sequenase™ version 2.0) and alpha-thio dNTPs. The thio dNTPs are used to resolve the "compression" artifacts like dITP is used for the Taq dye-terminator methods. The results with this system are such that bands vary in intensity about 10-fold.

Wayne Barnes has published a protocol for dye-terminator sequencing with FY modified polymerases and Mn^{2+} (Scientech Corp. St. Louis, MO). Bands are more uniform with this method varying about 4.5-fold at most.

Fluorescein-12 ddNTPs that have a linker length of 12 atoms and Biotin-11 ddNTPs that have a linker length of 11 atoms are available (Dupont NEN, Wilmington, DE). These labeled ddNTPs are described as useful in sequencing reactions.

ABI PRISM disclose dichlororhodamine dyes linked to terminators by propargyl/ethylene oxide/amino ("EO") linkers eight atoms in length for sequencing (Rosenblum, B.B., Lee, L.G., Spurgeon, S.L., Khan, S.H., Menchen, S.M., Heiner, C.R., and Chen, S.M., *Nucleic Acids Res.* 25(22):4500-4504, 1997).

Cyanine dyes have been utilized in dye terminators for sequencing (Lee et al., *Nucleic Acids Res.*, 20(10):2471, 1992).

SUMMARY OF THE INVENTION

The present invention provides novel dideoxy dye-labeled terminators which are useful in a number of biological processes, including providing uniform band intensities and the resolution of dye-induced compression artifacts in DNA sequencing. The dideoxy dye-labeled terminators of the present invention are particularly well suited for use with DNA polymerases that are thermostable or which contain an altered dNMP binding site (Tabor et al., U.S. Patent No. 5,614,365). Use of the terminators of the present invention for sequencing does not require the use of nucleotide analogs such as dITP or alpha-thio nucleotides to eliminate dye-induced compression artifacts. Applicant has surprisingly found that there is a strong correlation between the length of the link between the dye molecule and the nucleotide and band uniformity, but little correlation between the type of dye (or other parameters) and band uniformity. Dye terminators with linkers of 10 or more atoms (extended linkers) up to 25 atoms (10, 11, 12.....25) when used in sequencing reactions produce bands in sequencing gels of significantly improved uniformity compared with dye terminators with linkers less than 10 atoms.

The dye termininators of the present invention with extended linkers typically are provided in groups of

four (ATGC) with or without a thermostable DNA polymerase and are especially useful in a method of sequence analysis.

In a first aspect, the invention features a kit for DNA sequencing having a first, second, third and fourth dye terminator molecule, each of the dye terminator molecules has a dye molecule, a linker of at least 10 atoms in length and either ddATP, ddCTP, ddGTP or ddTTP as a mono or tri-phosphate and a thermostable DNA polymerase.

By "dye molecule" is meant any molecule that has a detectable emission spectrum, including but not limited to fluorescein, rhodamine, texas red, eosin, lissamine, coumarin, cyanine, and derivatives of these molecules. Dyes also include energy transfer dyes each comprising a donor and an acceptor dye.

By "linker" is meant a chain of at least 10 atoms comprising carbon, nitrogen, and oxygen which links the dye molecule with the dideoxynucleotide. The chain may also contain substituted carbon or sulfur. Linkage typically occurs at the aromatic base moiety of the nucleotide. The first two atoms of the linker attached to the base are typically joined in a triple bond.

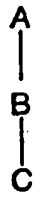
By "substituted carbon " is meant that one or more hydrogens are replaced with a substitute group such as, but not limited to, hydroxyl, cyano, alkoxy, oxygen, sulfur, nitroxy, halogen, $-N(CH_3)_2$, amino, and -SH.

By "thermostable DNA polymerase" is meant a DNA polymerase has a half-life of greater than 5 minutes at 90°C. Such polymerases include, but are not limited to,

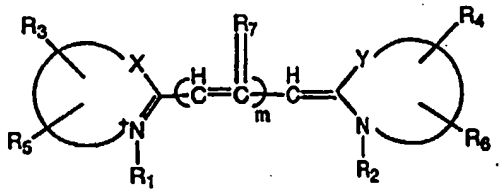
DNA polymerases encoded by *Thermus aquaticus*, *Thermus thermophilus*, *Thermus flavus*, *Thermococcus littoralis*, *Pyrococcus furiosus*, *Thermotoga maritima*, and *Thermotoga neapolitana*.

In a preferred embodiment the thermostable DNA polymerase has an altered dNMP binding site so as to improve the incorporation of dideoxynucleotides relative to the natural polymerase. A DNA polymerase with an altered dNMP binding site does not discriminate significantly between dideoxynucleotides and deoxynucleotides. The chance of incorporating a dideoxynucleotide is approximately the same as that of a deoxynucleotide or at least 1/10 the efficiency of a deoxynucleotide.

In a second aspect the invention features a compound of formula (I)



A is a cyanine dye of the structure

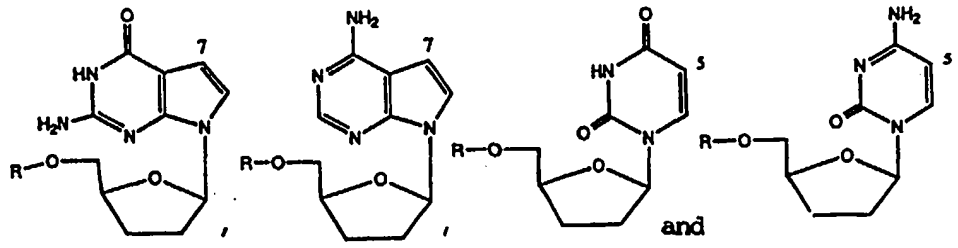


wherein the curved lines represent carbon atoms necessary for the formulation of cyanine dyes; X and Y are selected from the group consisting of O, S, and

CH_3-C-CH_3; m is an integer selected from the group consisting of 1, 2, 3, and 4; R1, R2, R3, R4, R5, R6, and R7 are independently selected from the group consisting of H, OH, CO_2H, sulfonic acid or sulfonate groups, esters, amides, ethers, alkyl or aryl groups, and B and one R1, R2, R3, R4, R5, R6 or R7 is B.

B is a linker of at least 10 atoms in length wherein the atoms are selected from the group consisting of carbon, nitrogen, oxygen, substituted carbon and sulfur and the linker is attached at one end to A and at the other end to C.

C is a dideoxynucleotide selected from the group consisting of:



and wherein the linker is covalently bonded to the dideoxynucleotide at position 7 for the purines (ddG, ddA) and at position 5 for the pyrimidines (ddT, ddC) and wherein r is a mono or tri-phosphate.

The term "sulfonic acid or sulfonate groups" refer to SO_3H groups or salts thereof.

The term "ester" refers to a chemical moiety with formula -(R)n-COOR', where R and R' are independently selected from the group consisting of saturated or

unsaturated alkyl and five-membered or six-membered aryl or heteroaryl moieties and where n is 0 or 1.

The term "amide" refers to a chemical substituent of formula -NHCOR, where R is selected from the group consisting of hydrogen, alkyl, hydroxyl, and five-membered or six-membered aryl or heteroaryl ring moieties, where the ring is optionally substituted with one or more substituents independently selected from the group consisting of alkyl, halogen, trihalomethyl, carboxylate, nitro, or ester.

The term "ether" refers to a chemical moiety with formula R-O-R' where R and R' are independently selected from the group consisting of saturated or unsaturated alkyl and five-membered or six-membered aryl or heteroaryl moieties and where n is 0 or 1.

The term "alkyl" refers to a straight-chain or branched aliphatic hydrocarbon. The alkyl group is preferably 1 to 10 carbons, more preferably a lower alkyl of from 1 to 7 carbons, and most preferably 1 to 4 carbons. Typical alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tertiary butyl, pentyl, hexyl and the like. The alkyl group may be substituted and some typical alkyl substituents include hydroxyl, cyano, alkoxy, oxygen, sulfur, nitroxy, halogen, $-N(CH_3)_2$, amino, and -SH.

The term "aryl" refers to an aromatic group which has at least one ring having a conjugated pi electron system and includes both carbocyclic aryl (e.g. phenyl) and heterocyclic aryl groups (e.g. pyridine). The term "carbocyclic" ref rs to a compound which contains one or

more covalently closed ring structures, and that the atoms forming the backbone of the ring are all carbon atoms. The term thus distinguishes carbocyclic from heterocyclic rings in which the ring backbone contains at least one atom which is different from carbon. The term "heteroarly" refers to an aryl group which contains at least one heterocyclic ring.

In a preferred embodiment the linker is selected from the group consisting of:

$-C{\equiv}C-CH_2-NH-CO-(CH_2)_5-NH-CO-$,

$-C{\equiv}C-CH_2-NH-CO-(CH_2)_9-NH-SO_2-$,

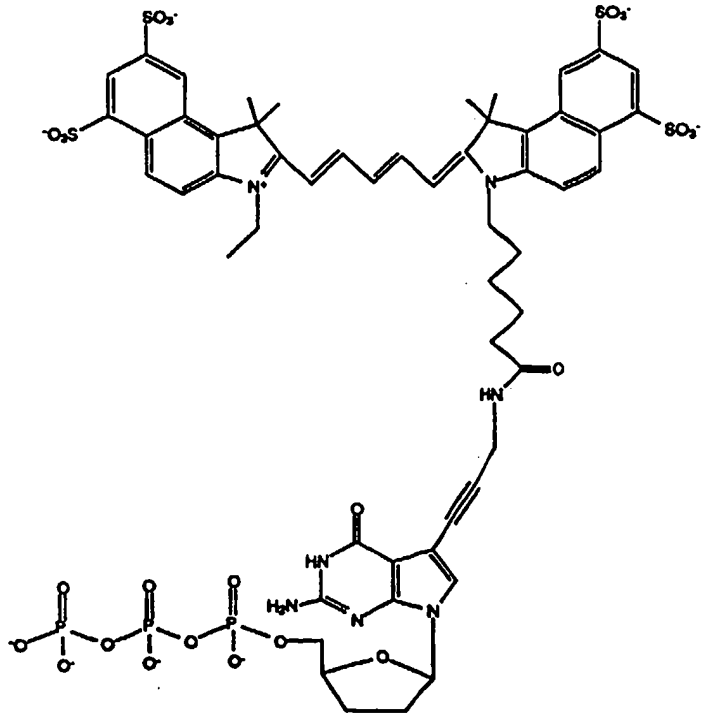
$-C{\equiv}C-CH_2-NH-CO-(CH_2)_{10}-NH-CO-$,

$-C{\equiv}C-CH_2-NH-CO-(CH_2)_5-$,

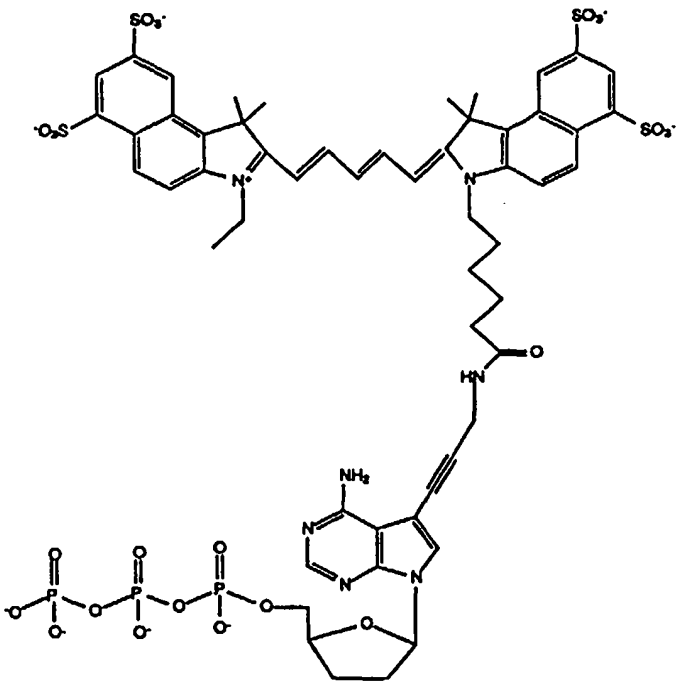
$-C{\equiv}C-CH_2-NH-CO-(CH_2)_5-NH-CO-(CH_2)_5-$, and

$-C{\equiv}C-CH_2-NH-CO-(CH_2)_5-NH-CO-(CH_2)_{10}-NH-CO-$

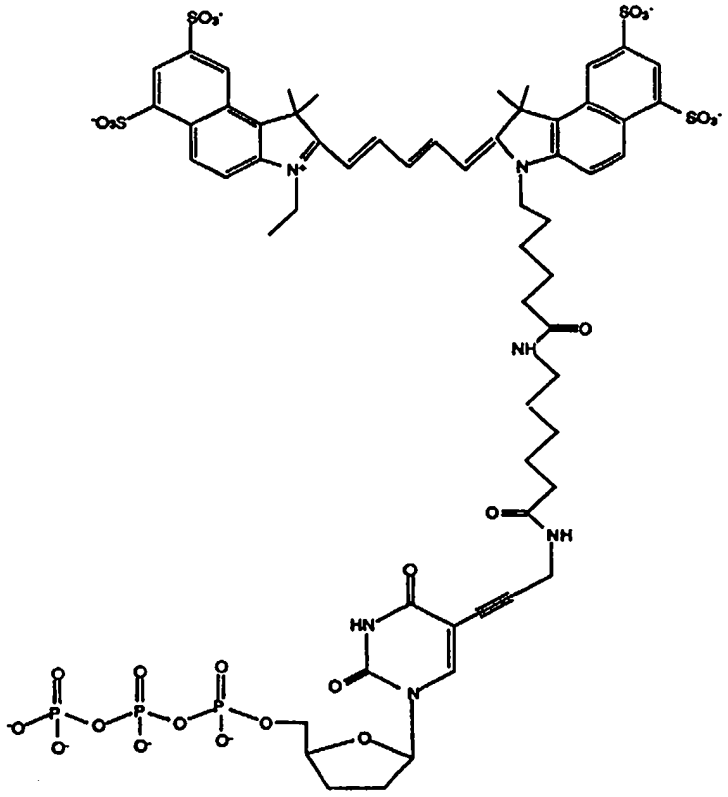
In preferred embodiments the dideoxy dye terminators are; a compound of the formula (II):



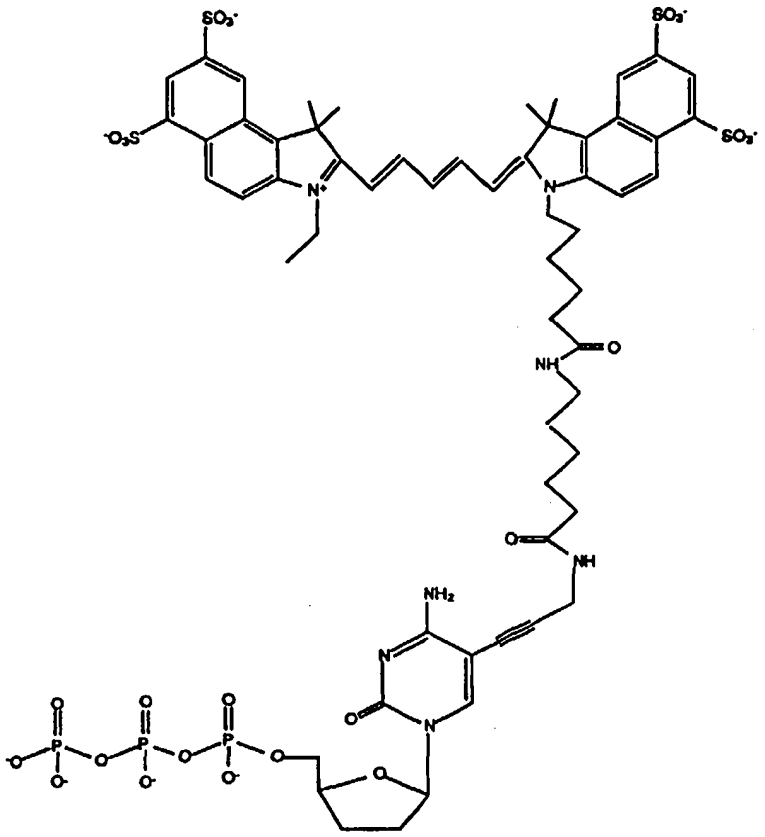
;a compound of the formula (III):



; a compound of the formula (IV):



;compound of the formula (V):



The Cy-5.5 ddGTP and ddCTP compounds have a linker of 10 atoms in length. The Cy-5.5 ddCTP and ddTTP compounds have a linker of 17 atoms in length.

In a third aspect the invention features a deoxyribonucleic acid sequence containing the compound of formula I, II, III ,IV or V.

In a preferred embodiment the invention features a kit for DNA sequencing comprising compounds of formula II, III, IV, and V.

In a further preferred embodiments the kit further has a thermostable DNA polymerase; the thermostable DNA polymerase has an altered dNMP binding site so as to improve the incorporation of dideoxynucleotides relative to the natural polymerase.

Applicant has surprisingly found that the one parameter that most strongly correlates with band uniformity is the length of the linker between the dye and the ddNTP. Applicant has found that by extending the linker length between the dye and the nucleotide for any dye:ddNTP combination to at least 10 atoms, that band uniformity is substantially improved and there are no dye-induced compression artifacts.

Thus, in a fourth aspect, the invention features a method for determining the nucleotide base sequence of a DNA molecule consisting of the steps of incubating a DNA molecule annealed with a primer molecule able to hybridize to the DNA molecule in a vessel containing a thermostable DNA polymerase, a dye terminator with a linker of at least 10 atoms between the dye and the nucleotide and separating DNA products of the incubating reaction according to size whereby at least a part of the nucleotide base sequence of the DNA molecule can be determined.

In preferred embodiments, the dye terminator is a compound of formula I, II, III, IV or V; the thermostable DNA polymerase has an altered dNMP binding site.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

All articles, publications and patents cited in this application are hereby incorporated by reference, in their entirety.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 presents DNA sequence data generated using M13mp18 containing a 115 bp SauAI fragment from lambda inserted a the BamHI site and Cy5.5 ddGTP, ddATP, ddTTP, and ddCTP dye terminators.

Fig. 2 is a graph of band intensity variability (rms) vs linker length (atoms).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following Examples are provided for further illustrating various aspects and embodiments of the present invention and are in no way intended to be limiting of the scope.

Example 1: Synthesis of dideoxy dye terminators Cy 5.5 dideoxynucleoside triphosphates

Dye terminators labeled with Cy5.5 were prepared from propargylaminodideoxynucleotids (Prober, J.M., Trainor, G.L., Dam, R.J., Hobbs, F.W., Robertson, C.W., Zagursky, R.J., Cocuzza, A.J., Jensen, M.A. and Baumeister, K., *Science* 238:336-41 (1987); U.S. Pat nt Nos. 5,242,796, 5,306,618, and 5,332,666) and "CyDye Fluorolink Cy5.5 mono reactive dye" product PA25501

(Amersham Life Science) to produce compounds II, III, IV, and V. In the case of ddG and ddA, the propargylaminonucleotide was directly reacted with the N-hydroxysuccinimidyl ester of the Cy5.5 dye. In the case of ddC and ddT, a longer linker was constructed by reacting the propargylaminonucleotide with the N-hydroxysuccinimidyl ester of N-trifluoroacetyl-6-aminocaproic acid followed by hydrolysis in aqueous ammonia of the trifluoroacetyl group. The resulting compound was then reacted with the N-hydroxysuccinimidyl ester of the Cy5.5 dye to give the 17-atom linker between the Cy 5.5 dye and the pyrimidine base.

In addition to Cy 5.5 dyes, those who practice the art would know how to identify and utilize other dyes, including other cyanine dyes, with the appropriate optical properties. Also, the construction and attachment of various linkers is well known in the art. Suitable reagents for linker construction include one or more compounds consisting of activated forms of amino-protected alkyl or aryl amino acids such as compounds of the formula $R\text{-}NH\text{-}(CH_2)_n\text{-}CO_2R'$ or $R\text{-}NH\text{-}(CH_2)_nX(CH_2)_m\text{-}CO_2R'$, where R is an acid- or base-labile protecting group, R' is a reactive ester or anhydride group, X is aryl, O, S, or NH, and where n and m are 0-12. Other linkers constructed by N- or O- or S- alkylation are also suitable. The exact linker length, of at least 10 atoms, for a specific dye and dideoxynucleotide combination can be determined empirically by monitoring band uniformity in DNA sequencing as described (see Example 3).

Example 2: Dye terminator cycle sequencing

DNA cycle sequencing was carried out using Thermo Sequenase™ DNA polymerase (Amersham, Cleveland, OH) and Cy5.5 dideoxy dye terminators using the following cycle sequencing protocol:

1. A master mix was prepared consisting of the following:

Template DNA	5.0μl
10X Reaction buffer (see below)	3.5μl
Primer, 2μM	1.0μl
Polymerase (see below)	2μ
H_2O	15.5μl
Total volume	27.0μl

10X Reaction Buffer:
150 mM Tris HCL pH 9.5
35mM $MgCl_2$

Polymerase: Thermo Sequenase™ DNA polymerase, 10U/μl, 0.0017U/μl, *Thermoplasma acidophilum* inorganic pyrophosphatase: 20mM Tris-HCl, pH 8.5, 1mM DTT, 0.1mM EDTA, 0.5% Tween-20, 0.5% Nonidet P-40 and 50% glycerol.

2. Four microcentrifuge tubes were labeled and 2 μl of Cy5.5 labeled ddG, ddA, ddT, ddC solution was added to each tube.

25:1 ddG Mix, 300 μM each of dGTP, dATP, dTTP & dCTP, 12μM Cy5.5 ddGTP
25:1 ddA Mix, 300 μM each of dGTP, dATP, dTTP & dCTP, 12μM Cy5.5 ddATP
25:1 ddT Mix, 300 μM each of dGTP, dATP, dTTP & dCTP, 12μM Cy5.5 ddTTP
25:1 ddC Mix, 300 μM each of dGTP, dATP, dTTP & dCTP, 12μM Cy5.5 ddCTP

3. Six μl of the master mix (from step 1) was aliquoted to each of the 4 tubes from step 2 above. Cycling was carried out as follows: 95°C (30 sec), 45-55°C (30 sec) and 72°C (60 sec) for 35 cycles then incubate at 72°C 5-7 minutes.

4. One μl of 8M ammonium acetate was added to each tube. Then 27 μl (approximately 3 times the reaction volume) of chilled 100% ethanol was added. Then mixture was mixed and placed on ice for 20 minutes to precipitate the DNA.

5. The mixture was centrifuged in a microcentrifuge (~12,000rpm) for 20-30 minutes at either room temperature or 4°C. The supernatant was removed and then 200 μl of 70% ethanol was added to wash the DNA pellet.

6. The mixture was again centrifuged for 5 minutes, the supernatant removed and the pellet dried (in a vacuum centrifuge) for 2-3 minutes.

7. Each pellet was resuspended in 6 μl of formamide loading dye (Amersham, Cleveland, OH), vortexed vigorously (10-20 sec) to ensure that all DNA was dissolved. The mixture was briefly centrifuged to collect the sample at the bottom of the tube.

8. Samples were heated to 70°C for 2-3 minutes to denature the DNA, then placed on ice.

9. Then 1.5-2 μl of the volume was loaded onto a lane of the sequencing gel, and the gel run on the MICRO Gene Blaster instrument (VGI).

For this sequence, the template DNA was M13mp18 containing a 115 bp Sau3AI fragment from bacteriophage lambda inserted at the BamHI site (product number US 70171 Amersham). The primer is the -40 Forward 23-mer universal primer (5'-GTTTTCCCAGTCACGACGTTGTA-3')(SEQ. ID. NO. 1). Results are shown in Figure 1.

Example 3: Correlation of linker length and band intensity variability

Sequencing reactions were carried out as described in example 2 with various dye molecules linked to dideoxynucleotides with linkers of various lengths (see Table 1). The labeled DNA products were then separated

on denaturing polyacrylamide gels and the labeled products were detected by fluorescence. The intensity of the bands is taken as the height of the peaks in a graph of fluorescence (in arbitrary units) against time. Typically, systematic variations in peak heights can be seen in graphs of peak heights plotted sequentially. These systematic variations in the peak heights can be modeled by least-square fitting to a second-order polynominal function. Dividing the peak height for each band by the value of the curve-fit polynomial function yields a normalized band intensity for each peak. Variation in these band intensities can be expressed as the square root of the variance $\sqrt{(n\Sigma x^2-(\Sigma x)^2/n^2)}$ of the normalized peak heights, which can typically have values between 0 and 1 with more variability represented by higher numbers (Fuller, C.W., *Comments* 16(3):1-8, 1989). This value is numerically equal to root-mean-square (RMS) value when 1.0 is subtracted from the normalized peak heights. These values are reported in Table 1 and graphed in Fig. 2. Variability of band intensities is significantly reduced when linkers of 10 or more atoms in length were used, resulting in sequence data that was easier to interpret accurately.

Table 1

	Base	Dye[a]	Linker Length[b]	Band Uniformity (rms)
1	T	Coumarin	5[c]	0.32
2	G	Lissamin	5[d]	0.77

3	G	R110	5[c]	0.34
4	A	R6G	5[c]	0.32
5	G	R6G	5[c]	0.57
6	C	ROX	5[c]	0.36
7	T	TMR	5[c]	0.47
8	A	TxR	5[d]	0.61
9	C	Eosin	6[e]	0.40
10	G	Cy3	10[i]	0.24
11	A	Cy5	10[i]	0.15
12	G	Cy5	10[i]	0.21
13	A	Cy5.5	10[i]	0.21
14	G	Cy5.5	10[i]	0.20
15	A	Fl	12[f]	0.16
16	C	Fl	12[f]	0.20
17	G	Fl	12[f]	0.17
18	T	Fl	12[f]	0.18
19	A	R6G	12[f]	0.13
20	T	R6G	12[f]	0.25
21	A	ROX	12[f]	0.21
22	T	ROX	12[f]	0.16
23	C	TMR	12[f]	0.26
24	G	TMR	12[f]	0.29
25	T	TMR	12[f]	0.37
26	A	TxR	16[g]	0.32
27	C	TxR	16[g]	0.24
28	G	TxR	16[g]	0.22
29	U	TxR	16[g]	0.24
30	A	Cy3-Cy5	17[j]	0.11
31	C	Cy3-Cy5	17[j]	0.16
32	G	Cy3-Cy5	17[j]	0.22

33	T	Cy3-Cy5	17[j]	0.11
34	C	Cy5	17[j]	0.14
35	T	Cy5	17[j]	0.10
36	C	Cy5.5	17[j]	0.20
37	T	Cy5.5	17[j]	0.18
38	A	Fl	17[h]	0.16
39	C	Fl	17[h]	0.24
40	G	Fl	17[h]	0.18
41	T	Fl	17[h]	0.25
42	T	Fl	24[k]	0.24

[a] Abbreviations for dyes: Fl, Carboxyfluorescein; R110, Rhodamine 110; R6G, Rhodamine 6G; ROX, Rhodamine X; TMR, tetramethylrhodamine; TXR, Texas Red (Molecular Probes). The dyes Cy3, Cy3.5, Cy5 and Cy5.5 were from Amersham Life Science, Cleveland, OH.

[b] Linker length is the number of atoms between the ring structure of the nucleoside base (A, C, G or T) and the ring structure of the dye.

Linker structures

[c] -C≡C-CH_2-NH-CO-

[d] -C≡C-CH_2-NH-SO_2-

[e] -C≡C-CH_2-NH-CS-NH-

[f] -C≡C-CH_2-NH-CO-$(CH_2)_5$-NH-CO-

[g] -C≡C-CH_2-NH-CO-$(CH_2)_9$-NH-SO_2-

[h] -C≡C-CH_2-NH-CO-$(CH_2)_{10}$-NH-CO-

[i] -C≡C-CH_2-NH-CO-$(CH_2)_5$-

[j] -C≡C-CH_2-NH-CO-$(CH_2)_5$-NH-CO-$(CH_2)_5$-

[k] -C≡C-CH_2-NH-CO-$(CH_2)_5$-NH-CO-$(CH_2)_{10}$-NH-CO-

CLAIMS

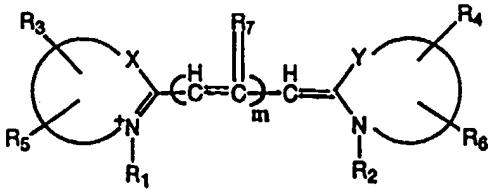
1. A kit for DNA sequencing comprising:

a first, second, third and fourth dye terminator molecule, each of the dye terminator molecules comprising a dye molecule, a linker of at least 10 atoms in length and either ddATP, ddCTP, ddGTP or ddTTP as a mono or tri-phosphate and a thermostable DNA polymerase.

2. The kit of claim 1, wherein said polymerase is a thermostable DNA polymerase that has an altered dNMP binding site so as to improve the incorporation of dideoxynucleotides relative to the natural polymerase.

3. A compound of formula (I):

wherein A is a cyanine dye of the structure

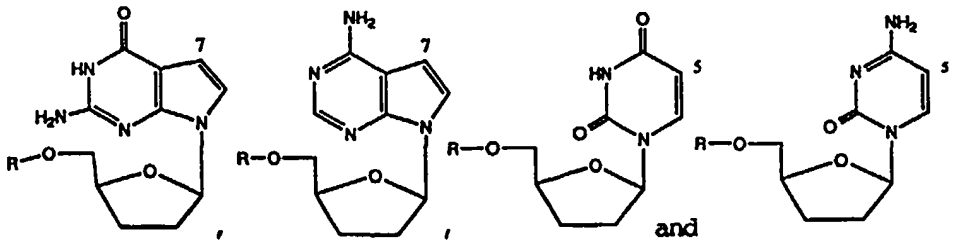


and the curved lines represent carbon atoms necessary for the formulation of cyanine dyes, X and Y are select d from the group consisting of O, S, and

CH_3-C-CH_3, m is an integer selected from the group consisting of 1, 2, 3, and 4, R1, R2, R3, R4, R5, R6 and R7 are independently selected from the group consisting of H, OH, CO_2H, sulfonic acid or sulfonate groups, esters, amides, ethers, alkyl or aryl groups and B, and one R1, R2, R3, R4, R5, R6 or R7 is B ;

B is a linker of at least 10 atoms in length wherein the atoms are selected from the group consisting of carbon, nitrogen, oxygen, substituted carbon, and sulfur and the linker is attached at one end to A and at the other end to C; and

C is a dideoxynucleotide selected from the group consisting of



wherein said linker is covalently bonded to said dideoxynucleotide at position 7 for ddA and ddG and at position 5 for ddC and ddT and wherein r is a mono or tri-phosphate.

4. The compound of claims 3, wherein said linker is selected from the group consisting of

$-C\equiv C-CH_2-NH-CO-(CH_2)_5-NH-CO-$,

$-C\equiv C-CH_2-NH-CO-(CH_2)_9-NH-SO_2-$,

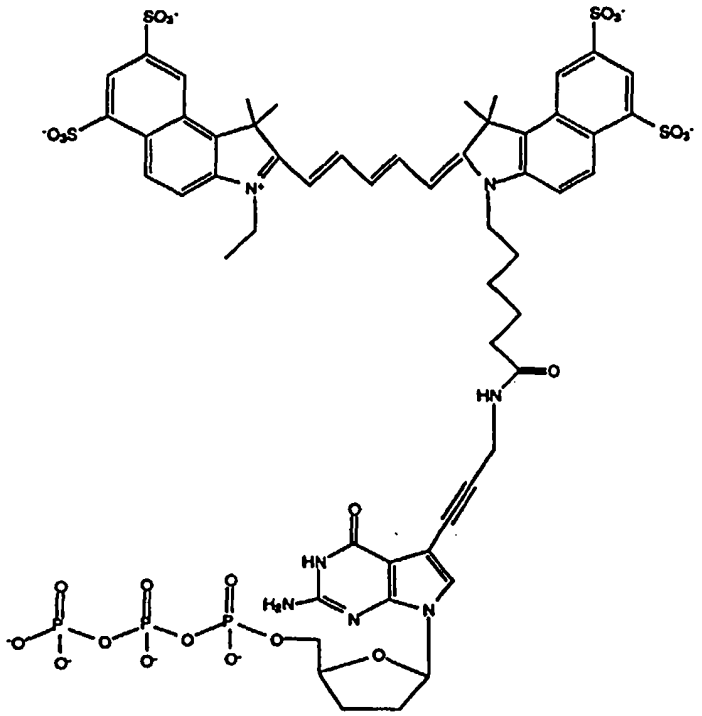
$-C\equiv C-CH_2-NH-CO-(CH_2)_{10}-NH-CO-$,

$-C\equiv C-CH_2-NH-CO-(CH_2)_5-$,

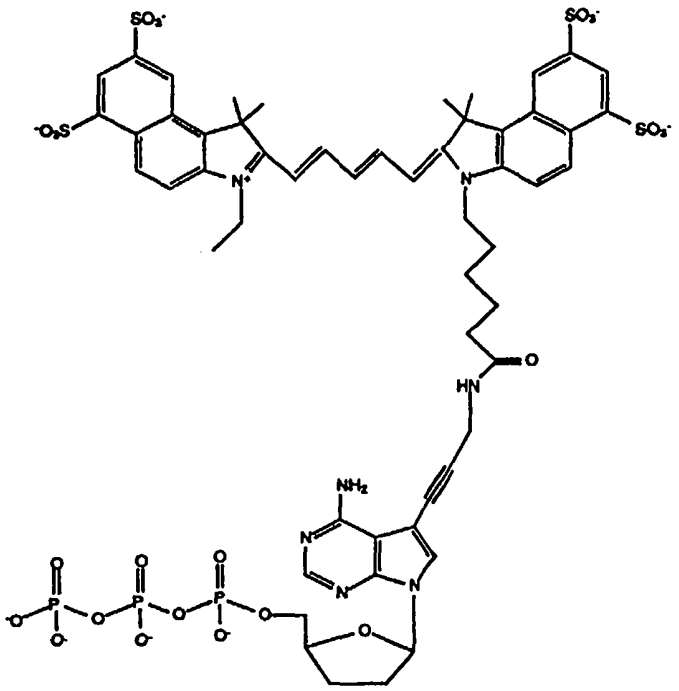
$-C\equiv C-CH_2-NH-CO-(CH_2)_5-NH-CO-(CH_2)_5-$, and

$-C\equiv C-CH_2-NH-CO-(CH_2)_5-NH-CO-(CH_2)_{10}-NH-CO-$.

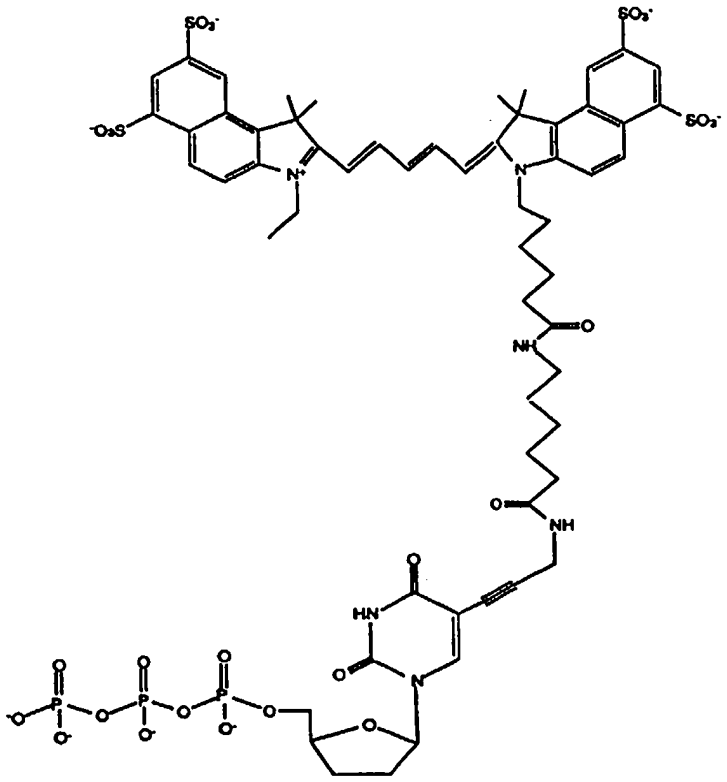
5. A compound of the formula (II):



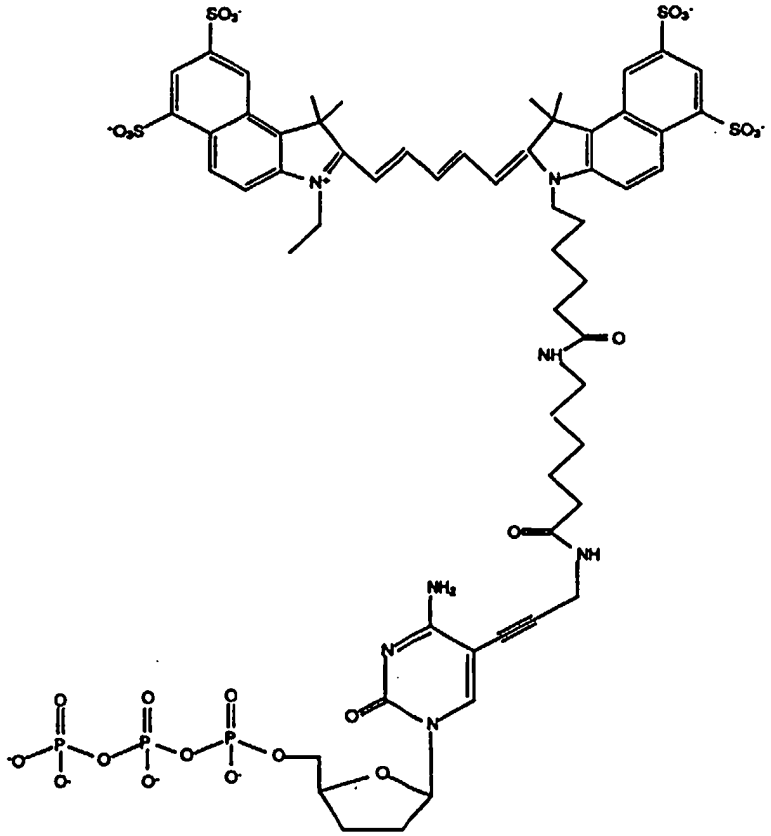
6. A compound of the formula (III):



7. A compound of the formula (IV):



8. A compound of the formula (V):



9. A deoxyribonucleic acid sequence containing the compound of formula I.

10. A deoxyribonucleic acid sequence containing the compound of formula II, III, IV, or V.

11. A kit for DNA sequencing comprising compounds of formula II, III, IV, and V.

12. The kit of claim 11, further comprising a thermostable DNA polymerase.

13. The kit of claim 12, wherein said polymerase is a thermostable DNA polymerase that has an altered dNMP binding site so as to improve the incorporation of dideoxynucleotides relative to the natural polymerase.

14. Method for determining the nucleotide base sequence of a DNA molecule comprising the steps of:

incubating a DNA molecule annealed with a primer molecule able to hybridize to said DNA molecule in a vessel containing a thermostable DNA polymerase, one of a set of four dye terminators with an linker of at least 10 atoms between the dye and the nucleotide and

separating DNA products of the incubating reaction according to size whereby at least a part of the nucleotide base sequence of said DNA molecule can be determined.

15. Method for determining the nucleotide base sequence of a DNA molecule comprising the steps of:

incubating a DNA molecule annealed with a primer molecule able to hybridize to said DNA molecule in a vessel containing a thermostable DNA polymerase, a compound of formula I and

separating DNA products of the incubating reaction according to size whereby at least a part of the nucleotide base sequence of said DNA molecule can be determined.

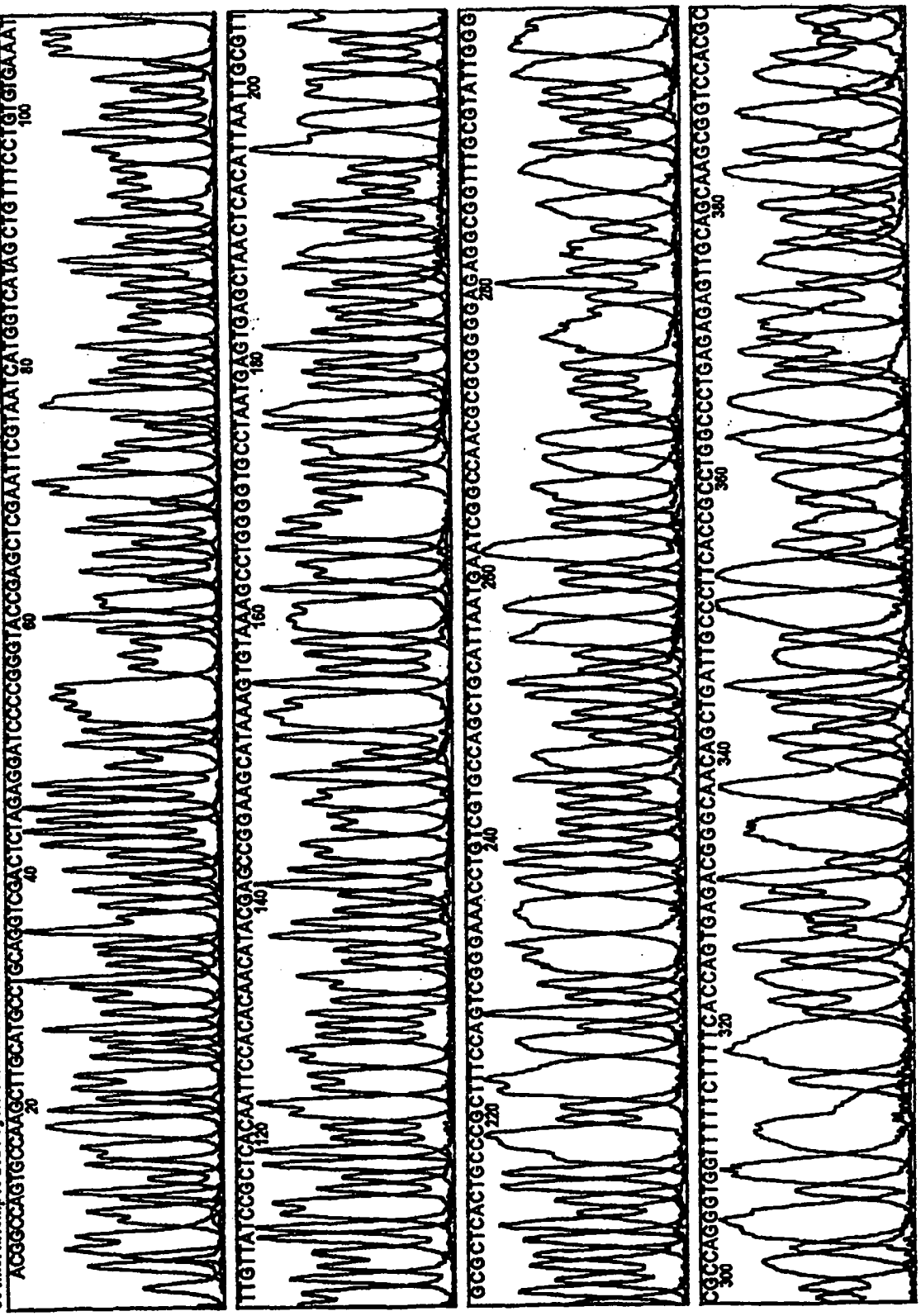
16. Method for determining the nucleotide base sequence of a DNA molecule comprising the steps of:

incubating a DNA molecule annealed with a primer molecule able to hybridize to said DNA molecule in a vessel containing a thermostable DNA, a compound of formula II, III, IV, or V and

separating DNA products of the incubating reaction according to size whereby at least a part of the nucleotide base sequence of said DNA molecule can be determined.

17. The method of any of claims 14, 15, or 16 wherein said polymerase is a thermostable DNA polymerase that has an altered dNMP binding site so as to improve the incorporation of dideoxynucleotides relative to the natural polymerase.

Figure 1


50 fmol M13mp18 DNA Cy5.5 Terminator dGTP
max A: 1205 C: 1012 G: 474 T: 537 First Sample 2110 Last: 7670
ACGGCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAAT
TTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTT
GCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGG
CGCCAGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGC
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380

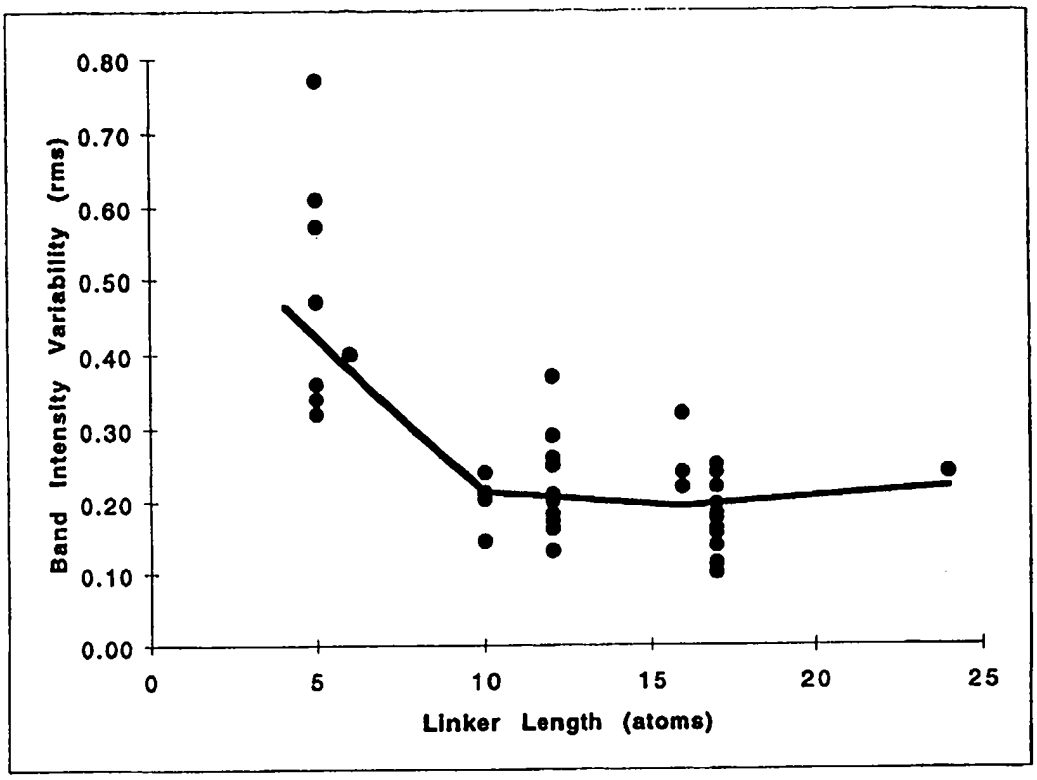

0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Band Intensity Variability (rms)
0
5
10
15
20
25
Linker Length (atoms)

FIGURE 2

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(I) APPLICANT: Kumar, Shiv
Nampalli, Stayam
McArdle, Bernard F.
Fuller, Carl W.

(ii) TITLE OF INVENTION: DIDEOXY DYE TERMINATORS

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Lyon & Lyon
(B) STREET: 633 West Fifth Street
Suite 4700
(C) CITY: Los Angeles
(D) STATE: California
(E) COUNTRY: U.S.A.
(F) ZIP: 90071-2066

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: IBM P.C. DOS 5.0
(D) SOFTWARE: FastSEQ for Windows 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: To Be Assigned
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Warburg, Richard J.
(B) REGISTRATION NUMBER: 32,327
(C) REFERENCE/DOCKET NUMBER: 225/219

(ix) TELECOMMUNICATION INFORMATION:

(A)	TELEPHONE:	(213) 489-1600
(B)	TELEFAX:	(213) 955-0440
(C)	TELEX:	67-3510

(2) INFORMATION FOR SEQ ID NO: 1:

(I) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	23 base pairs
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GTTTTCCCAG TCACGACGTT GTA 23

Other embodiments are within the following claims.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02104

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL : 435/6, 91.2, 194; 530/350; 536/22.1, 23.1, 26.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2, 194; 530/350; 536/22.1, 23.1, 26.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	EVANGELISTA et al. Characterization of fluorescent nucleoside triphosphates by capillary electrophoresis with laser-induced fluorescence detection: Action of alkaline phosphatase and DNA polymerase. Analytical Biochemistry. 1996, Vol. 235, pages 89-97, see entire document.	3,9 ---- 1,2,4-8,10-17
Y	US 5,614,365 A (TABOR et al) 25 March 1997, see entire document.	1-17
Y,P	US 5,795,762 A (ABRAMSON et al) 18 August 1998, see entire document.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"B" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 APRIL 1999	12 MAY 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JEFFREY FREDMAN JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02104

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HARALAMBIDIS et al. Preparation of base-modified nucleosides suitable for non-radioactive label attachment and their incorporation into synthetic oligodeoxyribonucleotides. Nucleic Acids Research. 1987, Vol. 15. No. 12, pages 4857-4876, see entire document.	1-17
Y	VANDER HORN et al. Thermo sequenase™ DNA polymerase and T. acidophilum pyrophosphatase: New thermostable enzymes for DNA sequencing. Biotechniques. 1997, Vol. 22. No. 4, pages 758-765, see entire document.	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02104

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6): C12Q 1/68; C12P 19/34; C12N 1/12; C07K 13/00; C07H 21/02, 21/04, 19/04

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAPLUS, REGISTRY, MEDLINE, BIOSIS
search terms: DNA, nucleic, oligo, polynucleotide, primer, probe, sequencing, polymerizing, polymerase, thermostable, adjust, alter, mutation, nucleotide, nucleoside, fluorescent, linker

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s)1-13, drawn to cyanine dye nucleotides for DNA sequencing and kits containing them.

Group II, claim(s) 14-17, drawn to methods of DNA sequencing.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims lack a special technical feature because the linking limitation between the two claims is a dye with a linker of at least 10 atoms between the dye and the nucleotide base. Nucleotides for use in DNA sequencing with this limitation are expressly taught by Evangelista et al (Anal. Biochem. (1996) 235:89-97). Therefore, there is no feature which distinguishes the claims over the prior art and consequently no special technical feature.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02104

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☒ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.